Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Characterization of genetic predisposition and autoantibody profile in atypical haemolytic–uraemic syndrome

Bahadur Singh Gurjar,[1,a] Tholu Manikanta Sriharsha,[2,b] Angika Bhasym,[3,4,a] Savit Prabhu,[5,b] Mamta Puraswani,[6,b] Priyanka Khandelwal,[6] Himanshi Saini,[6] Savita Saini,[6] Anita Kamra Verma,[7] Priyadarshini Chatterjee,[3] Prasenjit Guchhait,[3] Vineeta Bal,[1,5] Anna George,[1] Satyajit Rath,[1,5,8] Arvind Sahu,[2] Amita Sharma,[6] Pankaj Hari,[6] Aditi Sinha[6] and Arvind Bagga[6]

[1]*National Institute of Immunology, New Delhi,* [2]*National Centre for Cell Science, S. P. Pune University Campus, Pune,* [3]*Regional Centre for Biotechnology, Faridabad,* [4]*Department of Biotechnology, Manipal Academy of Higher Education, Manipal,* [5]*Paediatric Biology Centre, Translational Health Science and Technology Institute, Faridabad,* [6]*Department of Paediatrics, All India Institute of Medical Sciences, New Delhi,* [7]*Kirori Mal College, University of Delhi, Delhi, and* [8]*Agharkar Research Institute, Pune, India*

doi:10.1111/imm.12916
Received 2 October 2017; revised 1 February 2018; accepted 2 February 2018.
[a]These authors contributed equally to the work.
[b]These authors contributed equally to the work.
Correspondence: Satyajit Rath, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi 110067, India.
Email: satyajit@nii.ac.in
Senior author: Satyajit Rath

Summary

We previously reported that Indian paediatric patients with atypical haemolytic–uraemic syndrome (aHUS) showed high frequencies of anti-complement factor H (FH) autoantibodies that are correlated with homozygous deletion of the genes for FH-related proteins 1 and 3 (FHR1 and FHR3) (*FHR1/3*$^{-/-}$). We now report that Indian paediatric aHUS patients without anti-FH autoantibodies also showed modestly higher frequencies of the *FHR1/3*$^{-/-}$ genotype. Further, when we characterized epitope specificities and binding avidities of anti-FH autoantibodies in aHUS patients, most anti-FH autoantibodies were directed towards the FH cell-surface anchoring polyanionic binding site-containing C-terminal short conservative regions (SCRs) 17–20 with higher binding avidities than for native FH. FH SCR17–20-binding anti-FH autoantibodies also bound the other cell-surface anchoring polyanionic binding site-containing region FH SCR5–8, at lower binding avidities. Anti-FH autoantibody avidities correlated with antibody titres. These anti-FH autoantibody characteristics did not differ between aHUS patients with or without the *FHR1/3*$^{-/-}$ genotype. Our data suggest a complex matrix of interactions between *FHR1-FHR3* deletion, immunomodulation and anti-FH autoantibodies in the aetiopathogenesis of aHUS.

Keywords: complement factor H autoantibodies; genetic predisposition to autoimmunity; haemolytic–uraemic syndrome.

Abbreviations: aHUS, atypical haemolytic–uraemic syndrome; AU, arbitrary units; CCP, complement control protein; ELISAs, enzyme-linked immunosorbent assays; FH, factor H; FHRs, FH-related genes; LDH, lactate dehydrogenase; MLPA, multiple ligation-dependent probe amplification; qPCR, quantitative real-time PCR; SCR, short conservative region; SEM, standard error of mean; STL, Shiga-like toxin

Introduction

Among the categories of haemolytic–uraemic syndrome (HUS) identified, apart from secondary HUS, are the bacterial Shiga-like toxin (SLT) -associated forms and the 'atypical' (aHUS) forms[1,2] in which disturbances of the complement pathway feature prominently.[3–5] Many mutations in complement pathway-related genes such as factor H (FH), FH-related proteins (FHRs), factor I, CD46, factor B and C3 have been reported to be associated with aHUS, especially in children.[3] In many reported series of paediatric aHUS, autoantibodies directed against FH have been identified and implicated in pathogenesis,[6,7] and the therapeutic utility of immunosuppression and plasma exchange has been shown.[8,9] Notably, the majority of aHUS patients with anti-FH autoantibodies have a deletion of the genes for the FH-related proteins 1 and 3 (FHR1 and FHR3), commonly as the result of being homozygous for a widespread ~ 83-kb deletion encompassing the *FHR1–FHR3* genes at chromosome 1q32.[10]

Anti-FH autoantibody levels in aHUS are negatively correlated with clinical outcome.[11] They are primarily of the IgG3 isotype,[12] and are thought to be mostly directed at the C-terminal cell-surface anchorage-related region of FH,[13–16] although greater epitope diversity has also been reported.[17,18] FH is a negative regulator of complement activation, and there is evidence from mouse models that specific abrogation of FH functions at cell surfaces by inhibiting its cell-binding, with preservation of its fluid-phase functions, leading to the typical histopathology of HUS.[19]

The mechanism by which the *FHR1–FHR3* deletion predisposes children to anti-FH autoantibody formation is unknown. FHR proteins appear to exist as homo- and hetero-dimeric species in which FHR1 is prominent, and competitively inhibit FH binding to cell surfaces.[20] There is also some evidence that FH and the FHRs can preferentially inhibit different steps of the complement cascade.[21] What role, if any, these FHR properties have on the levels and characteristics of anti-FH autoantibodies generated in children homozygous for the *FHR1–FHR3* deletion is currently unclear.

Paediatric aHUS patients with anti-FH autoantibodies typically do not show the presence of other autoantibodies,[11,22] suggesting an antigen-specific break in immune tolerance. However, the *FHR1–FHR3* deletion has also been reported to be associated with increased risk of systemic lupus erythematosus,[15,23] and with decreased risks for age-related macular degeneration and IgA nephropathy,[24,25] possibly indicating more global immune function alterations, although these remain uncharacterized.

On this background, we have begun to examine a number of these poorly understood issues. Using an Indian paediatric aHUS series showing high frequency of anti-FH autoantibodies and associated *FHR1–FHR3* deletion,[8] we report data here suggesting that the permissive role of the *FHR1–FHR3* deletion in aHUS may not be restricted to anti-FH autoantibody generation, that anti-FH autoantibodies show variable avidity correlated with their levels but are epitopically restricted, and that patients with or without the *FHR1–FHR3* deletion have similar properties of their anti-FH autoantibodies. Together, our data provide insights into the complexity of interactions between *FHR1–FHR3* deletion, immunomodulation and anti-FH autoantibodies in aHUS.

Materials and methods

Patients and healthy volunteers

Patients below 18 years of age with suspected aHUS in the absence of diarrhoeal prodrome in the preceding fortnight from centres across India were included from 2007 onwards. HUS was diagnosed in the presence of acute kidney injury, anaemia with schistocytes, thrombocytopenia and high serum levels of lactate dehydrogenase.[5,26] Patients with features of thrombotic microangiopathy secondary to human immunodeficiency virus, septicaemia, disseminated intravascular coagulation and systemic lupus erythematosus were excluded. Blood was collected from an antecubital vein into heparinized tubes and plasma and cells were separated. Cells were centrifuged on a Ficoll-Hypaque gradient, peripheral blood mononuclear cells were separated, and cells and DNA were stored at −80° until assay.

All work was reviewed and approved by the institutional human ethics committees of the relevant participating institutions, and informed consent for all participants was obtained.

DNA purification, multiple ligation-dependent probe amplification and real-time quantitative PCR assays

Genomic DNA was extracted from peripheral blood mononuclear cells (Qiagen, Hilden, Germany) and purified (Genomic DNA isolation kit; Advanced Microdevices, Ambala, India) using commercial reagents. An appropriate commercial multiple ligation-dependent probe amplification (MLPA) assay was used for the estimation of heterozygous and homozygous deletions of *FHR1* and *FHR3* genes according to the manufacturers' instructions using SALSA MLPA probe mix P236-A2 ARMD mix 1 (lot 1109) and probe mix P236-A3 ARMD mix 1 (lot 0811) (MRC-Holland, Amsterdam, the Netherlands), using 125 ng of denatured genomic DNA for each reaction and sequence-specific probes for exons of *FH*. The *FHR3*, *FHR1*, *FHR2* and *FHR5* genes were used for PCR amplification, followed by analysis on a capillary electrophoresis system (ABI

PRISM 3130 Genetic analyser), and calculation of relative peak heights and areas for each target (Coffalyser, MRC-Holland) with normalization using known samples and identification of peak heights or areas between 40 and 70% as heterozygous deletion genotypes.[8]

For real-time quantitative PCR assays, intron-3 genomic regions in both *FHR1* and *FHR3* loci[27] were amplified (Power SYBR Green PCR Master Mix; AB Systems, Waltham, MA) and 0·2 μM of the indicated primers with requisite *Taq* DNA polymerase, reaction buffer (New England Biolabs, Ipswich, MA), magnesium chloride and dNTPs (Promega, Madison, WI). The assay was performed using a real-time PCR system (Eppendorf Mastercycler ep realplex 4). Cycle parameters: initial denaturation at 95°, 10 min, followed by 40 cycles of 15 seconds denaturation at 95°, 30 seconds annealing at 66° (*FHR1*) or 68° (*FHR3*) or 61° (*BETA-ACTIN*) followed by 30 seconds extension at 66° (final extension, 7 min).[27] All reactions were performed in triplicates. The values obtained for *FHR1* and *FHR3* gene copy numbers were normalized to the endogenous control gene *beta actin* and quantified relative to the copy number of control samples using the ΔΔCT method.[28]

Primers used[27]:

FHR1; forward 5′-ACATCTCCAATTTAGATCCTTTGATTAACCA-3′,

reverse 5′-GCATTTTCTTAGTGAATAAGCAAAGATTTAAAAACA-3′;

FHR3; forward, 5′-ACCGCTCTGAGATCCCAGCATG-3′,

reverse, 5′-GGTCCGTTGGCAAAACAAGTTGAC-3′;

BETA-ACTIN; forward, 5′-GACCTGACTGACTACCTCATG-3′,

reverse, 5′-AGCAGCCGTGGCCATCTCTT-3′.

Conventional in-gel PCR assays were also performed (Veriti 96-well Thermal cycler, Applied Biosystems, Foster City, CA) with the same primers and conditions to ensure that amplified DNA bands of expected sizes could be detected. PCR products were run on 2·5% agarose gel electrophoresis. Predicted sizes were 100 bp, 120 bp and 150 bp for the *FHR1*, *FHR3* and *BETA-ACTIN* genes, respectively.

Cloning, expression and purification of FH truncation mutants

The construction of FH truncation mutants [short conservative region 1–4 (SCR1–4), SCR5–8, SCR9–12, SCR13–16 and SCR17–20] was achieved by PCR amplification of the required domains from the FH cDNA (OriGene, Rockville, MD) and cloning of these amplicons into pET28 or pET29 vectors. The SCR1–4, SCR5–8, SCR9–12 and SCR13–16 constructs were cloned in pET 28 at the *Nco*I and *Xho*I sites, whereas the SCR17–20 construct was cloned in pET 29 at the *Nde*I and *Xho*I sites. The primers were designed so that each mutant started with the first Cys of the starting domain (except for SCR1–4, which started with Glu) and ended with the last residue of the interdomain linker. The clones generated were verified by DNA sequencing (ABI 3730 DNA analyzer; Applied Biosystems). Appropriate primer sets were used for PCR amplification of the CCP domain regions (see Supplementary material, Table S1). Expression of all the truncation mutants was accomplished by transformation of the clones into *Escherichia coli* BL21 cells and induction of expression with 1 mM isopropyl 1-thio-β-D-galactopyranoside as detailed elsewhere.[29,30] All expressed truncation mutants were found in inclusion bodies, and were purified as described earlier[29,31] using Ni-NTA resin (Qiagen) in the presence of urea followed by elution with imidazole. The eluted mutants were then refolded by the rapid dilution method[32] and further purified on Superose12 gel filtration columns (Pharmacia, Stockholm, Sweden) to obtain the monodisperse species, confirmed as > 95% pure by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (see Supplementary material, Fig. S1).

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assay (ELISA) plates (Microlon high-binding flat-bottomed 96-well plates; Greiner, Bahlingen, Germany) were coated with FH from human plasma (0·5 μg/ml; Sigma-Aldrich, St Louis, MO), blocked, serum-titrated (1 : 100–1 : 100 000) and bound antibodies were detected with peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich) followed by *o*-phenylenediamine dihydrochloride (Sigma-Aldrich), and colour read and analysed (Tecan microplate reader, Magellan software). A reference patient serum pool was used to calibrate the assay and to calculate anti-FH autoantibody concentrations in arbitrary units (AU)/ml (for examples, see Supplementary material, Fig. S2).[33] The reference serum pool used for quantification was calibrated with the Dragon-Durey laboratory reference standard widely described in the literature.[33] The detection limit was 200 AU/ml.

Relative avidities and epitope specificities of anti-FH autoantibodies were examined by using titrating concentrations of either native FH protein or various recombinant FH fragments to inhibit antibody binding to plate-coated native FH. Serum dilutions that gave detectable but submaximal binding to native FH were determined for each serum sample, were mixed with various inhibitor concentrations and added to FH-coated plates for ELISAs. The extent of inhibition was estimated, and 50% inhibitory concentrations were calculated where indicated.

The FH-SCR17–20 fragment was used to coat plates instead of native FH protein where indicated.

To detect FH-containing immune complexes, plates were coated with 2 μg/ml of mouse anti-human

complement factor H monoclonal antibody (MCA509G[34]; AbD Serotec, Hercules, CA), or, in a few assays, polyclonal anti-FH antibody (Bio-Rad, Hercules, CA). Control and test sera were titrated as shown, and bound FH-containing immune complexes were specifically detected using peroxidase-conjugated monoclonal mouse anti-human IgG (05-4220; Invitrogen, Calrsbad, CA) or polyclonal anti-human IgG (Sigma).

Statistical analysis

Comparisons were performed using, where appropriate as indicated, analysis of variance, the Mann–Whitney *U*-test with no assumption of normality, or a two-tailed Student's *t*-test with unequal variance, as appropriate, and a *P*-value of $< 0{\cdot}05$ was considered significant.

Results

Multiple ligation-dependent probe amplification, quantitative PCR and end-point PCR assays for detection of FHR1 and FHR3 gene deletions

Real-time quantitative PCR (qPCR) assays showed single peaks in the melting curves and a reliable quantitative titration for both the *FHR1* and *FHR3* genes and the *beta-actin* gene (see Supplementary material, Fig. S3). MLPA assays showed, as expected based on literature,[10] that *FHR1* and *FHR3* deletions were found together; individuals showed either homozygous deletions, heterozygous deletions or undeleted status for both genes (see Supplementary material, Fig. S4), and these genotypes could be correctly detected by the qPCR assays (see Supplementary material, Fig. S4). The end-point PCR assays, when analysed on agarose gels, showed bands of expected sizes, and could clearly distinguish between DNA samples showing homozygous *FHR1* and *FHR3* deletion versus undeleted controls (see Supplementary material, Fig. S5).

We next compared the MLPA, the qPCR and the end-point PCR assays in DNA samples of aHUS patients and family members ($n = 143$) for consistency in detecting the *FHR1* and *FHR3* genotype. Almost all instances of homozygous *FHR1* and *FHR3* deletion were detected with complete concordance in all three methods, and heterozygous *FHR1* and *FHR3* deletions were also detected with substantial accuracy by the qPCR assays (Table 1).

When we tested another set of 357 healthy human blood donor samples, we found 35 (9·8%) to be of the −/− genotype, confirming our earlier report of presence in the asymptomatic Indian population at a frequency similar to that reported elsewhere.[8]

Table 1. Comparison of multiple ligation-dependent probe amplification (MLPA), quantitative PCR (qPCR) and end-point PCR methods for CHFR1 and CFHR3 genotyping

		Genotype		
Gene	Method	+/+	+/−	−/−
CFHR1	MLPA	85	46	12
	qPCR	85	41	17
	End-point PCR	86	58	
CFHR3	MLPA	79	51	13
	qPCR	79	50	14
	End-point PCR	78	49	

High frequencies of FHR1 and FHR3 gene deletions in anti-FH autoantibody-based aHUS categories

The bulk of the association between aHUS and the *FHR1* and *FHR3* deletion-homozygous genotype has been shown to be in the group of aHUS patients bearing anti-FH autoantibodies.[6,35] However, most of these patient series have been relatively small. We therefore estimated the genotypes for *FHR1* and *FHR3* gene deletion in paediatric aHUS patients either with ($n = 96$) or without ($n = 68$) anti-FH autoantibodies, and compared them with aHUS patient family members ($n = 65$) and unrelated healthy adult volunteers ($n = 84$), as reported earlier.[8] As expected, aHUS patients with anti-FH autoantibodies were homozygous for *FHR1* and *FHR3* deletion with very high frequencies (Table 2). However, even patients with no detectable anti-FH autoantibodies were homozygous for *FHR1* and *FHR3* deletion with frequencies higher than those in healthy volunteers (Table 2; $P < 0{\cdot}02$), suggesting the possibility (subject to caveats explored below) that the homozygous *FHR1–3*-deletion genotype may also make some contribution to aHUS through anti-FH autoantibody-independent pathways.

Table 2. Frequencies of FHR1 and FHR3 deletion in various groups

Patient group	Gene	−/− (%)	−/+ (%)	+/+ (%)	Total
aHUS; with anti-FH antibodies	*FHR1*	78 (81%)	11 (12%)	7 (7%)	96
	FHR3	72 (75%)	19 (20%)	5 (5%)	
aHUS; without anti-FH antibodies	*FHR1*	15 (22%)	25 (36%)	28 (41·17%)	68
	FHR3	13 (19%)	25 (37%)	30 (44·11%)	
aHUS patient family members[1]	*FHR1*	30 (46%)	29 (45%)	6 (9%)	65
	FHR3	28 (43%)	33 (51%)	4 (6%)	
Healthy volunteers[1]	*FHR1*	8 (10%)	30 (36%)	46 (55%)	84
	FHR3	8 (10%)	30 (36%)	46 (55%)	

Abbreviations: aHUS, atypical haemolytic–uraemic syndrome; FH, factor H; FHR, FH-related gene.
[1]Data from ref.[8]

FH-containing immune complexes are not detected in anti-FH autoantibody-negative aHUS patients

As the frequency of homozygous *FHR1* and *FHR3* deletion in paediatric aHUS patients with no anti-FH autoantibodies appeared to be high, it was necessary to examine if these patients simply had levels of anti-FH autoantibodies too low to be detected in the assay used. The quantified levels of anti-FH autoantibodies were not different between the two groups (Fig. 1a). Nonetheless, even in the absence of free detectable anti-FH antibodies, FH-containing immune complexes would be expected to be present. We therefore tested some sera from four patient groups, with or without either *FHR1* deletion and/or anti-FH autoantibodies, for FH-containing immune complexes. However, those tested patients who did not show detectable free anti-FH autoantibodies did not show detectable FH-containing immune complexes either, regardless of their genotype (Fig. 1d and e). As expected, patients with free anti-FH antibodies also showed strong signals for FH-containing immune complexes (Fig. 1a and b).

The epitope recognized by the plate-coated MCA509G monoclonal antibody, secreted by the OX-24 clone, involves SCR5 of FH.[34] Although some interference of MCA509G with FH-bound autoantibodies may occur, it

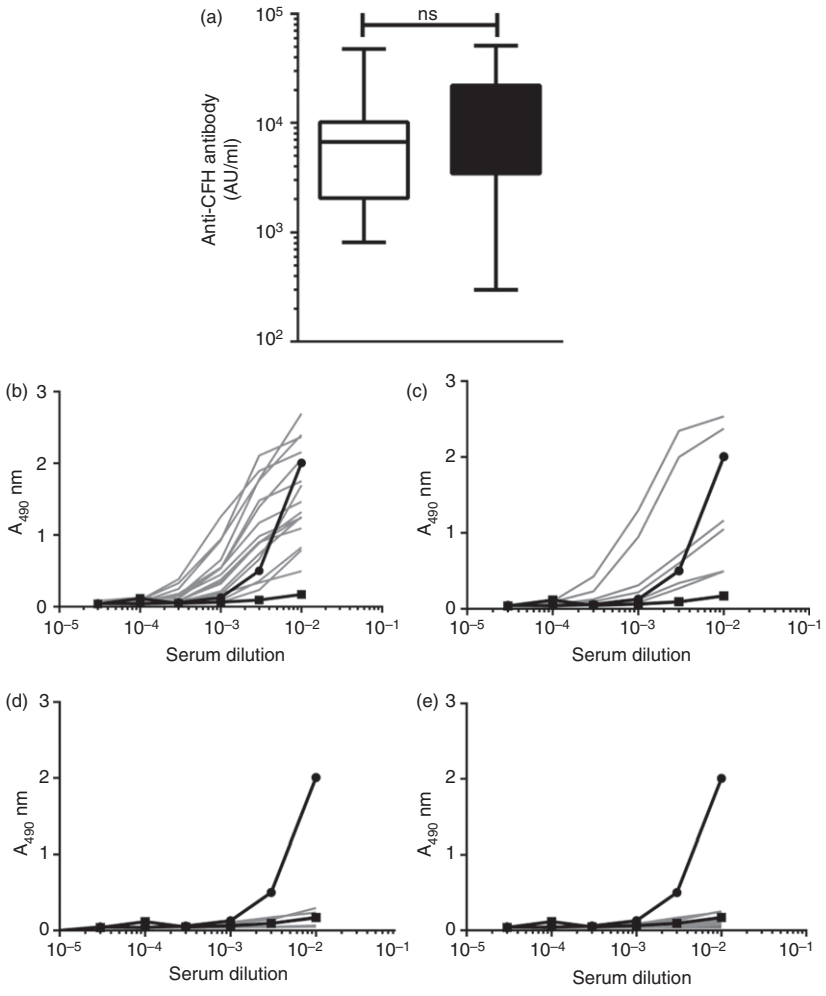

Figure 1. Anti-factor H (FH) autoantibody levels and FH-containing immune complexes in sera of atypical haemolytic–uraemic syndrome (aHUS) patients with or without a homozygous deletion genotype for FH-related genes 1 (FHR1) and and 3 (FHR3). (a) Anti-FH antibody levels in sera of aHUS patients with (open; $n = 15$) or without (filled; $n = 6$) a homozygous deletion genotype for *FHR1* and *FHR3* as indicated. Levels were estimated by ELISA as described and quantified using a reference pool of patient sera. (b–e) FH-containing immune complexes were detected as described in sera from four groups of patients; anti-FH antibody-positive with a homozygous deletion genotype (b), anti-FH antibody-positive without a homozygous deletion genotype (c), anti-FH antibody-negative with a homozygous deletion genotype (d), and anti-FH antibody-negative without a homozygous deletion genotype (e). All assays (b–e) also used a reference pool of patient sera as a positive control (filled circles) and of healthy volunteers (filled squares) as a negative control. Representative plots for at least three independent experiments are shown.

is unlikely that it would shift FH from the immune complex state to the free state. This possibility is supported by the fact that FH-immune complex assays using either MCA509G or a polyclonal anti-FH antibody did not show any differences, indicating that displacement of bound autoantibody by the plate-coated anti-FH antibodies was unlikely to be a major concern for assay validity.

Hence, these data further supported the possibility that the homozygous *FHR1–3*-deletion genotype may make some modest contribution to the pathogenesis of aHUS through anti-FH autoantibody-independent pathways.

Analysis of epitope specificity and avidity of anti-FH autoantibodies

We next examined the epitope specificity of serum anti-FH autoantibodies in paediatric aHUS patients homozygous for deletion of *FHR1* and *FHR3* genes. Five recombinant FH fragments, FH-SCR1–4, FH-SCR5–8, FH-SCR9–12, FH-SCR13–16 and FH-SCR17–20, were used. The relative sizes of these fragments were as expected (see Supplementary material, Fig. S1). We chose to use inhibition ELISAs with these fragments to ensure detection and analysis of native FH-relevant antibodies and to provide definitive evidence for cross-reactivity where appropriate. The binding of anti-FH autoantibodies in the reference pool of patient sera to native solid-phase FH was not detectably inhibited by FH-SCR1–4, FH-SCR9–12, or FH-SCR13–16 (Fig. 2a). Aqueous-phase native FH inhibited binding well as expected, and the C-terminal fragment, FH-SCR17–20, inhibited FH-binding of serum autoantibodies well, with an IC_{50} value (0·1 μM) higher than that for native FH (0·21 μM) (Fig. 2a). Notably, FH-SCR5–8 also substantially inhibited anti-FH autoantibody binding to native FH, albeit at lower relative avidity (1·17 μM) (Fig. 2a).

This raised the possibility that anti-FH autoantibodies in aHUS sera were cross-reactive between FH-SCR17–20 and FH-SCR5–8. We tested this by using the FH-SCR-17–20 fragment as the solid-phase, and using native FH, FH-SCR17–20, or FH-SCR5–8 to inhibit the binding of autoantibodies in the pool of aHUS patient sera. Once again, aqueous-phase native FH inhibited binding to FH-SCR17–20, as did FH-SCR17–20 itself, with comparable efficiency (Fig. 2b). Further, FH-SCR5–8 also efficiently inhibited autoantibody binding to FH-SCR17–20,

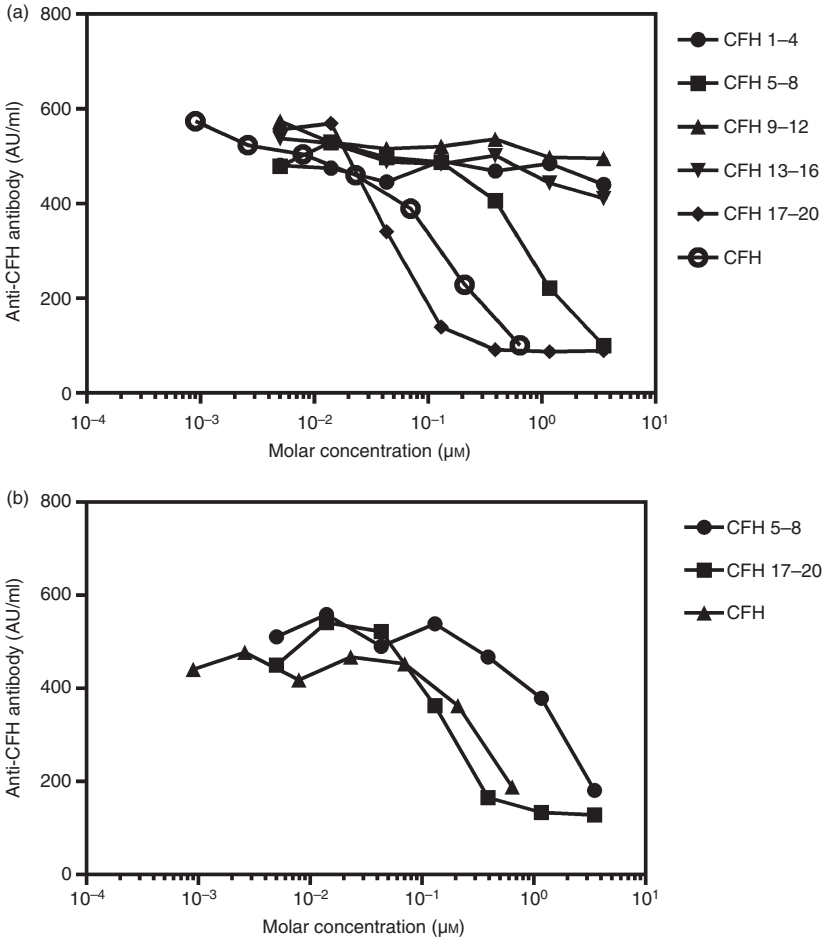

Figure 2. Epitope specificity mapping of anti-faactor H (FH) autoantibodies. Appropriate dilutions of reference pool of atypical haemolytic–uraemic syndrome (aHUS) patient sera were incubated with titrating concentrations of either native FH or recombinant FH fragments short conservative region 1–4 (SCR1–4), SCR5–8, SCR9–12, SCR13–16 or SCR17–20 as shown, before being assayed on plates coated with either native FH (a) or SCR17–20 (b).

although with lower efficiency (Fig. 2b). Hence, the bulk of anti-FH autoantibodies in a pool of aHUS patient sera recognized FH-SCR17–20 with high affinity and cross-reacted with FH-SCR5–8 at low affinity.

Epitope specificity and avidity of anti-FH autoantibodies in paediatric aHUS patients

We next examined the epitope specificities of anti-FH autoantibodies in aHUS patients, both in those who were homozygous for *FHR1* and *FHR3* deletion ($n = 15$) and in those who did not show this deletion genotype ($n = 6$). In the majority of instances of both genotypes, high concentrations of either native FH or FH-SCR17–20 caused over 80% inhibition of binding to solid-phase native FH (Fig. 3a). High concentration of FH-SCR5–8 also led to over 60% inhibition in most instances (Fig. 3a). Lower inhibitor concentrations led to variable reductions in solid-phase FH binding (Fig. 3a), indicating variable anti-FH autoantibody avidity. Relative avidities,

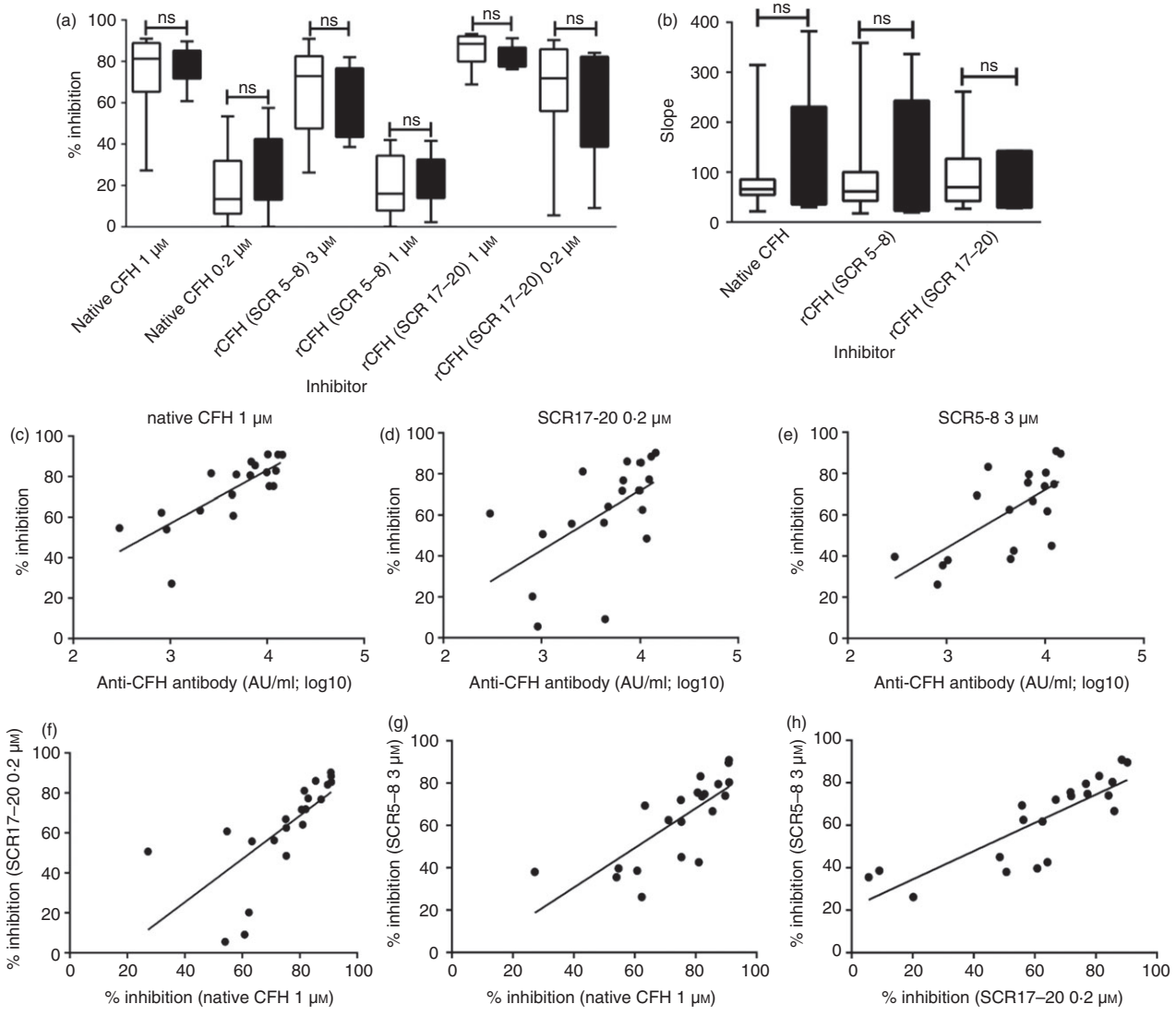

Figure 3. Epitope specificity and relative avidity in anti-factor H (FH) autoantibodies in atypical haemolytic–uraemic syndrome (aHUS) patients with or without homozygous deletion of FH-related genes 1 (FHR1) and 3 (FHR3). Appropriate serum dilutions were pre-incubated with either native FH (1 μM or 0·2 μM), or recombinant short conservative region 5–8 (SCR5–8) (3 μM or 1 μM) or SCR17–20 (1 μM or 0·2 μM) for 30 min before being transferred to native FH-coated plates for ELISA. The extent of inhibition by each concentration of inhibitor was calculated. (a) Data are shown as median and interquartile range for aHUS patients with ($n = 15$; white boxes) or without ($n = 6$) homozygous deletion of *FHR1* and *FHR3*. (b) Slope values calculated for each inhibitor for the two concentrations used as median and interquartile range for aHUS patients with ($n = 15$; white boxes) or without ($n = 6$) homozygous deletion of *FHR1* and *FHR3*. (c–e) Correlations between anti-FH antibody levels and extent of inhibition by the indicated inhibitor are shown. For all three, $P < 0{\cdot}05$. (f–h) Correlations between extent of inhibition by the various indicated inhibitors are shown. For all three, $P < 0{\cdot}05$.

as indicated by the slopes of the lines defined by the two concentrations used of each inhibitor tested, did not differ between the patient groups that were or were not homozygous for *FHR1* and *FHR3* deletion (Fig. 3b).

The anti-FH autoantibody levels were well-correlated with the extent of inhibition observed at a given inhibitor concentration (Fig. 3c–e), indicating that higher autoantibody levels were associated with higher avidity. Native FH, SCR17–20 and SCR5–8 were well correlated with each other with regard to the extent of inhibition caused in all sera tested (Fig. 3f–h), further suggesting the prominence of antibodies recognizing the cross-reactive epitopes on SCR17–20 and SCR5–8.

We also looked for indications of correlations, if any, between epitope specificity and avidity of anti-FH autoantibodies and clinical patterns in 20 of these patients with a median age of 8·8 years (range 6·5–10 years). In this small group of patients, the avidity or epitope specificity did not show any significant associations with disease severity (haemoglobin, platelet count, serum creatinine, duration of oliguria, serum lactate dehydrogenase) or adverse outcomes, or between patients with ($n = 6$) or without ($n = 14$) relapse.

Discussion

We have attempted to probe some of the puzzles regarding aHUS using a series of paediatric aHUS patients. Using qPCR and PCR approaches to detecting the homozygous 83-kb deletion encompassing *FHR1* and *FHR3* genes in a large paediatric aHUS series, we find that the deletion is modestly but clearly over-represented even in aHUS patients that have no detectable anti-FH autoantibodies or immune complexes, suggesting possible anti-FH autoantibody-independent pathways connecting the deletion to aHUS. We have characterized anti-FH autoantibody affinities and epitope specificities, and find highly variable affinities and relatively conserved epitope specificities for the C-terminus of FH. However, we also find that these C-terminal-specific anti-FH autoantibodies also cross-react at low affinity with the mid-portion of the FH molecule, suggesting possible pathways for anti-FH autoantibody-mediated modulation of FH function.

Although MLPA is currently used as the reference standard for detection of the *FHR1–FHR3* deletion, we found that the optimized qPCR detected both homozygous and heterozygous genotypes equally well. In fact, a simple in-gel PCR assay for FHR1 detected the homozygous genotype quite reliably. Together, these PCR-based assays may well be of use for easier detection of the *FHR1–FHR3* deletion.

The *FHR1–FHR3* deletion has been classically reported to be associated with the anti-FH autoantibody-positive form of aHUS,[1,36] although there has been some indication of occasional aHUS patients with the *FHR1–FHR3* deletion but without anti-FH autoantibodies.[6] Our data, based on substantial numbers of both anti-FH autoantibody-positive and -negative aHUS patients, provide evidence that *FHR1–FHR3* deletion also occurs in anti-FH autoantibody-negative aHUS at a higher frequency than that in the normal population. It is well recognized that the *FHR1–FHR3* deletion occurs in the healthy population as a polymorphism.[3] In our population, ~ 9% of healthy individuals (without aHUS) had a homozygous *FHR1–FHR3* deletion, whereas ~ 20% of anti-FH antibody-negative aHUS patients and ~ 75% of anti-FH antibody-positive aHUS patients had a homozygous *FHR1–FHR3* deletion. One possibility was that the lack of detection of anti-FH autoantibodies in at least some of these aHUS patients could be a consequence of low antibody levels that were mostly bound to circulating FH, leading to antibody-mediated modulation of FH function in the absence of detectable anti-FH antibodies. We examined this possibility by assaying for FH-containing immune complexes. However, we were unable to detect such immune complexes in the sera of anti-FH antibody-negative aHUS patients homozygous for the *FHR1–FHR3* deletion. As the epitope recognized by the plate-coated MCA509G monoclonal antibody involves FH-SCR5,[34] and the major anti-FH autoantibodies in our patient series do cross-react with FH-SCR5–8, our data show that those antibodies also bind with higher avidity to FH-SCR17–20, suggesting that interference of MCA509G with FH-bound autoantibodies is unlikely to shift FH from the immune complex state to the free state, consistent with the fact that FH-immune complex assays using either MCA509G or a polyclonal anti-FH antibody did not show any differences.

These data suggest that the *FHR1–FHR3* deletion can also predispose to paediatric aHUS via additional pathways independent of the known major pathway of a break of FH-specific B-cell tolerance. Whether such an aHUS sub-group has additional risk factors and/or distinct clinical behaviours needs to be explored.

In the present data, we have not explored the presence of a number of other mutations in complement-related genes that are known in HUS, including the genes for FH and monocyte chemoattractant protein[37,38] Hence, it is possible that the anti-FH-negative aHUS patients associated with the modest increase of *FHR1–FHR3* deletion that we observe are in fact, related to the presence of such mutations, although this would still not explain the increased frequency of the *FHR1–FHR3* deletion in such sub-groups.

It is noteworthy that the *FHR1–FHR3* deletion is also associated in various ways with other anti-FH autoantibody-independent immuno-inflammatory diseases such as systemic lupus erythematosus,[4] IgA nephropathy[5] and age-related macular degeneration.[27] Also, the *FHR1–FHR3* deletion is not sufficient in itself to give rise to long-lasting anti-FH autoantibodies, as evidenced by the

wide prevalence of this deletion without concomitant anti-FH antibodies in healthy populations.[3] Together, these data suggest that it is necessary to examine immunomodulation by the *FHR1–FHR3* deletion in both FH-specific and more general ways.

Anti-FH autoantibodies in aHUS patients have also been shown to cross-react with C-terminal regions of FHR1,[8] although the functional significance of this finding in human disease is unclear because these anti-FH autoantibodies are associated with a homozygous *FHR1–FHR3* deletion genotype.

In addition to socio-geographic settings, our series of paediatric aHUS patients differs from other reports in terms of the high prevalence of anti-FH autoantibody-positive patients.[7] We therefore sought to characterize the anti-FH antibodies in these patients in further detail. Although most previous studies have identified the C-terminus of FH to be the binding site for aHUS-associated anti-FH autoantibodies,[8,10,11] there have been reports of recognition of the N-terminal and central regions of FH as well.[11] Our data show that anti-FH autoantibodies in Indian paediatric aHUS patient sera dominantly if not exclusively recognize the C-terminal SCR17–20 region of FH. Notably, although our data show the presence of sero-reactivity to the mid-region SCR5–8 of FH, we find that most autoantibodies binding to FH-SCR5–8 are those that bind to the C-terminal SCR17–20. In fact, the affinity of these autoantibodies to FH-SCR5–8 is uniformly lower than their affinity to FH-SCR17–20. Hence, FH-SCR17–20 is likely to be the dominant immunogenic epitope for anti-FH autoantibodies in paediatric aHUS.

Interestingly, FH-SCR19–20 and SCR6–8 are reported to be involved in binding to cell surface glycoproteins.[13] Anti-FH antibodies are known to have functional consequences; they block binding of factor H to cell surfaces and hence affect complement regulation.[10] FH functions to regulate complement activity both in the fluid phase and on cell surfaces.[15,37] In fact, a complete deletion of the FH gene and function in mice leads to membranoproliferative glomerulonephritis[19] whereas partial complementation with a C-terminal-deficient fluid-phase active form of HUS in these mice changes the disease to an HUS-like syndrome. In this context, the dominance of anti-FH autoantibodies directed in cross-reactive fashion to the cell-surface anchorage regions of FH that we find may be particularly relevant.

Autoimmune antibody-mediated disease pathogenesis for some target antigens has been correlated with antibody affinity, although such associations have been variably observed.[39,40] For some autoantigens and autoantibodies, the immune complexes themselves deposit in glomeruli and contribute to disease,[12,16,18] and low-affinity antibodies may generate persistently circulating immune complexes.[41,42] This is not likely to be relevant for aHUS, as there is no evidence of immune complex deposition contributing to the disease.[6] Other autoimmune targets, such as the acetylcholine receptor, are cell-surface molecules, and effective binding may be achieved despite low antibody affinity. In this context, we were interested to investigate the possible roles that anti-FH autoantibody affinity may play in aHUS, and we now show an assay for both FH-specific and epitope-specific relative affinity. Using this assay, we find a wide variety of average relative affinities in the anti-FH autoantibodies, and autoantibody concentrations do not correlate with affinities. These assays now enable the future examination of whether clinical parameters in aHUS show any associations with either affinity and/or with epitope specificities of anti-FH autoantibodies. These will be critical issues in understanding the complex connections between *FHR1/3* deletion and immuno-inflammatory diseases.

Acknowledgements

This work was supported in part by grants from the Department of Biotechnology (DBT), Government of India (GoI) (to AG # BT/PR10954/BRB/10/625/2008 and # BT/PR12849/MED/15/35/2009; to VB # BT/PR-10284/Med/29/59/2007 and # BT/PR14420/Med/29/213/2010; to SR # BT/PR-14592/BRB/10/858/2010), from the Department of Science and Technology (DST), GoI (to V.B. # SR/SO/HS-0005/2011; to SR # SB/SO/HS/210/2013) and from the Indo-French Centre for the Promotion of Advanced Research (to AB # IPC/A/Network-1/2017/537). The National Institute of Immunology, the National Centre for Cell Sciences, the Translational Health Science and Technology Institute and the Regional Centre for Biotechnology are funded by grants-in-aid from the DBT, GoI. None of the funding sources had any involvement in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication. BSG and AB performed experiments, analysed the data, and helped write the manuscript. TMS, SB and MP developed critical tools and analysed data. MS and SS developed critical methodologies and helped collect the data. PK, AS, PH and AB conceptualized the approach, did the critical clinical components of the work, supervised the data collection, and analysed the data. AKV, PC and PG provided crucial conceptual inputs. VB, AG, SR, AS and AB conceptualized the approach, designed the experiments, analysed the data, and wrote the manuscript. All authors read, edited and approved the final manuscript.

Disclosures

SR is a non-executive director of Ahammune Biosciences Private Limited, Pune, India, and a member of the scientific advisory boards of Curadev Pharma Private Limited,

NOIDA, India, and Mynvax Private Limited, Bangalore, India. Other authors have no financial interests to declare.

References

1 Anagnou NP, Papanicolaou N, Fessas P. Recurrent attacks of hemolytic uremic syndrome. *Haematologia (Budap)* 1991; **24**:101–5.

2 Tarr PI, Gordon CA, Chandler WL. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet*, 2005:1073–86.

3 Warwicker P, Goodship TH, Donne RL, Pirson Y, Nicholls A, Ward RM *et al.* Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* 1998; **53**:836–44.

4 Noris M, Remuzzi G. Hemolytic uremic syndrome. *J Am Soc Nephrol* 2005; **16**:1035–50.

5 Goodship TH. Atypical HUS and complement dysregulation. *J Am Soc Nephrol* 2006; **17**:1775–6.

6 Dragon-Durey MA, Loirat C, Cloarec S, Macher MA, Blouin J, Nivet H *et al.* Anti-Factor H autoantibodies associated with atypical hemolytic uremic syndrome. *J Am Soc Nephrol* 2005; **16**:555–63.

7 Skerka C, Jozsi M, Zipfel PF, Dragon-Durey MA, Fremeaux-Bacchi V. Autoantibodies in haemolytic uraemic syndrome (HUS). *Thromb Haemost* 2009; **101**:227–32.

8 Sinha A, Gulati A, Saini S, Blanc C, Gupta A, Gurjar BS *et al.* Prompt plasma exchanges and immunosuppressive treatment improves the outcomes of anti-factor H autoantibody-associated hemolytic uremic syndrome in children. *Kidney Int* 2014; **85**:1151–60.

9 Khandelwal P, Sinha A, Hari P, Bagga A. Plasma exchanges and immunosuppression for anti-complement factor H associated hemolytic uremic syndrome. *Indian Pediatr* 2014; **51**:833–5.

10 Zipfel PF, Edey M, Heinen S, Jozsi M, Richter H, Misselwitz J *et al.* Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. *PLoS Genet* 2007; **3**:e41.

11 Dragon-Durey MA, Sethi SK, Bagga A, Blanc C, Blouin J, Ranchin B *et al.* Clinical features of anti-factor H autoantibody-associated hemolytic uremic syndrome. *J Am Soc Nephrol* 2010; **21**:2180–7.

12 Durey MA, Sinha A, Togarsimalemath SK, Bagga A. Anti-complement-factor H-associated glomerulopathies. *Nat Rev Nephrol* 2016; **12**:563–78.

13 Oppermann M, Manuelian T, Jozsi M, Brandt E, Jokiranta TS, Heinen S *et al.* The C-terminus of complement regulator Factor H mediates target recognition: evidence for a compact conformation of the native protein. *Clin Exp Immunol* 2006; **144**:342–52.

14 Jozsi M, Oppermann M, Lambris JD, Zipfel PF. The C-terminus of complement factor H is essential for host cell protection. *Mol Immunol* 2007; **44**:2697–706.

15 Wu H, Boackle SA, Hanvivadhanakul P, Ulgiati D, Grossman JM, Lee Y *et al.* Association of a common complement receptor 2 haplotype with increased risk of systemic lupus erythematosus. *Proc Natl Acad Sci USA* 2007; **104**:3961–6.

16 Trojnar E, Jozsi M, Uray K, Csuka D, Szilagyi A, Milosevic D *et al.* Analysis of linear antibody epitopes on factor H and CFHR1 using sera of patients with autoimmune atypical hemolytic uremic syndrome. *Front Immunol* 2017; **8**:302.

17 Jozsi M, Licht C, Strobel S, Zipfel SL, Richter H, Heinen S *et al.* Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. *Blood* 2008; **111**:1512–4.

18 Blanc C, Roumenina LT, Ashraf Y, Hyvarinen S, Sethi SK, Ranchin B *et al.* Overall neutralization of complement factor H by autoantibodies in the acute phase of the autoimmune form of atypical hemolytic uremic syndrome. *J Immunol* 2012; **189**:3528–37.

19 Pickering MC, de Jorge EG, Martinez-Barricarte R, Recalde S, Garcia-Layana A, Rose KL *et al.* Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. *J Exp Med* 2007; **204**:1249–56.

20 Goicoechea de Jorge E, Caesar JJ, Malik TH, Patel M, Colledge M, Johnson S *et al.* Dimerization of complement factor H-related proteins modulates complement activation *in vivo*. *Proc Natl Acad Sci USA* 2013; **110**:4685–90.

21 Heinen S, Hartmann A, Lauer N, Wiehl U, Dahse HM, Schirmer S *et al.* Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. *Blood* 2009; **114**:2439–47.

22 Westra D, Volokhina E, van der Heijden E, Vos A, Huigen M, Jansen J *et al.* Genetic disorders in complement (regulating) genes in patients with atypical haemolytic uraemic syndrome (aHUS). *Nephrol Dial Transplant* 2010; **25**:2195–202.

23 Johanneson B, Lima G, von Salome J, Alarcon-Segovia D, Alarcon-Riquelme ME. A major susceptibility locus for systemic lupus erythemathosus maps to chromosome 1q31. *Am J Hum Genet* 2002; **71**:1060–71.

24 Hughes AE, Orr N, Esfandiary H, Diaz-Torres M, Goodship T, Chakravarthy U. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nat Genet* 2006; **38**:1173–7.

25 Gharavi AG, Kiryluk K, Choi M, Li Y, Hou P, Xie J *et al.* Genome-wide association study identifies susceptibility loci for IgA nephropathy. *Nat Genet* 2011; **43**:321–7.

26 Campistol JM, Arias M, Ariceta G, Blasco M, Espinosa M, Grinyo JM *et al.* An update for atypical haemolytic uraemic syndrome: diagnosis and treatment. A consensus document. *Nefrologia* 2013; **33**:27–45.

27 Kubista KE, Tosakulwong N, Wu Y, Ryu E, Roeder JL, Hecker LA *et al.* Copy number variation in the complement factor H-related genes and age-related macular degeneration. *Mol Vis* 2011; **17**:2080–92.

28 Chang S, Chen W, Yang J. Another formula for calculating the gene change rate in real-time RT-PCR. *Mol Biol Rep* 2009; **36**:2165–8.

29 Yadav VN, Pyaram K, Mullick J, Sahu A. Identification of hot spots in the variola virus complement inhibitor (SPICE) for human complement regulation. *J Virol* 2008; **82**:3283–94.

30 Arico M, Burgio GR. Mononuclear cell derangement. *Pediatr Hematol Oncol* 1989; **6**:277–82.

31 Gautam AK, Panse Y, Ghosh P, Reza MJ, Mullick J, Sahu A. Mutational analysis of Kaposica reveals that bridging of MG2 and CUB domains of target protein is crucial for the cofactor activity of RCA proteins. *Proc Natl Acad Sci USA* 2015; **112**:12794–9.

32 White J, Lukacik P, Esser D, Steward M, Giddings N, Bright JR *et al.* Biological activity, membrane-targeting modification, and crystallization of soluble human decay accelerating factor expressed in E. coli. *Protein Sci* 2004; **13**:2406–15.

33 Watson R, Lindner S, Bordereau P, Hunze EM, Tak F, Ngo S *et al.* Standardisation of the factor H autoantibody assay. *Immunobiology* 2014; **219**:9–16.

34 Johnson SA, Williams JM, Hakobyan S, Richards A, Perkins SJ, Marchbank KJ *et al.* Impact of compound heterozygous complement factor H mutations on development of atypical hemolytic uremic syndrome-A pedigree revisited. *Mol Immunol* 2010; **47**:1585–91.

35 Strobel S, Abarrategui-Garrido C, Fariza-Requejo E, Seeberger H, Sanchez-Corral P, Jozsi M. Factor H-related protein 1 neutralizes anti-factor H autoantibodies in autoimmune hemolytic uremic syndrome. *Kidney Int* 2011; **80**:397–404.

36 Foltyn Zadura A, Zipfel PF, Bokarewa MI, Sturfelt G, Jonsen A, Nilsson SC *et al.* Factor H autoantibodies and deletion of Complement Factor H-Related protein-1 in rheumatic diseases in comparison to atypical hemolytic uremic syndrome. *Arthritis Res Ther* 2012; **14**:R185.

37 Cruzado JM, de Cordoba SR, Melilli E, Bestard O, Rama I, Sanchez-Corral P *et al.* Successful renal transplantation in a patient with atypical hemolytic uremic syndrome carrying mutations in both factor I and MCP. *Am J Transplant* 2009; **9**:1477–83.

38 Bresin E, Rurali E, Caprioli J, Sanchez-Corral P, Fremeaux-Bacchi V, Rodriguez de Cordoba S *et al.* Combined complement gene mutations in atypical hemolytic uremic syndrome influence clinical phenotype. *J Am Soc Nephrol* 2013; **24**:475–86.

39 Devey ME, Bleasdale K, Lee S, Rath S. Determination of the functional affinity of IgG1 and IgG4 antibodies to tetanus toxoid by isotype-specific solid-phase assays. *J Immunol Methods* 1988; **106**:119–25.

40 Rath S, Stanley CM, Steward MW. An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity heterogeneity. *J Immunol Methods* 1988; **106**:245–9.

41 Steward MW, Katz FE, West NJ. The role of low affinity antibody in immune complex disease. The quantity of anti-DNA antibodies in NZB/W F1 hybrid mice. *Clin Exp Immunol* 1975; **21**:121–30.

42 Boes M. Role of natural and immune IgM antibodies in immune responses. *Mol Immunol* 2000; **37**:1141–9.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primers used for the construction of truncated CFH fragments.

Figure S1 SDS–PAGE analysis of CFH and its truncation mutants.

Figure S2 Immunoassay of serum anti-CFH autoantibodies.

Figure S3 Quantitative PCRs for the human *CFHR1*, *CFHR3* and *BETA-ACTIN* genes.

Figure S4 Multiple ligation-dependent probe amplification and quantitative PCR analyses for *CFHR1*, *CFHR3* and *BETA-ACTIN* genes.

